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Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens

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Abstract

Ammonium chloride (NH_4Cl) was usually used as a model ammonia source to simulate ammonia inhibition during anaerobic digestion (AD) of nitrogen-rich feedstocks. However, ammonia in AD originates mainly from degradation of proteins, urea and nucleic acids, which is distinct from NH_4Cl. Thus, in this study, the inhibitory effect of a "natural" ammonia source (urea) and NH_4Cl, on four pure methanogenic strains (aceticlastic: *Methanosarcina thermophila*, *Methanosarcina barkeri*; hydrogenotrophic: *Methanoculleus bourgensis*, *Methanoculleus thermophilus*), was assessed under mesophilic (37°C) and thermophilic (55°C) conditions. The results showed that urea hydrolysis increased pH significantly to unsuitable levels for methanogenic growth, while NH_4Cl had a negligible effect on pH. After adjusting initial pH to 7 and 8, urea was significantly stronger inhibitor with longer lag phases to methanogenesis compared to NH_4Cl. Overall, urea seems to be more toxic on both aceticlastic and hydrogenotrophic methanogens compared to NH_4Cl under the same total and free ammonia levels.

Keywords

Ammonia inhibition; Ammonium chloride; Anaerobic digestion; Pure strain; Urea.

1 Introduction

Biogas (a mixture of CH_4 and CO_2) is an attractive renewable energy (Holm-Nielsen et al., 2009), which is formed during anaerobic digestion (AD) of different biomasses. As one of the most promising and widely used green technologies, AD is a complex biological process with different microorganisms involved, which can reduce the waste pollution and offset part of the energy usage (Chynoweth et al., 2001). However, it is reported that some potential substrates are toxic to AD process by inhibiting the microorganisms' activity (Chen et al., 2008). Among these substrates, nitrogen-rich substrates stand out, due to the ammonia formation during their degradation. A low ammonia concentration (< 200 mg NH_4^+-N L^{-1}) is beneficial to AD process; nevertheless, relatively high ammonia levels (> 2000 mg NH_4^+-N L^{-1}) would inhibit AD, causing instability and even process failure (Liu and Sung, 2002). Total ammonia (TAN) in aqueous solutions is the sum of ammonium ions (NH_4^+) and free ammonia (FAN, NH_3). The NH_4^+ and NH_3 exist in an equilibrium (Eq. (1)), which is affected by the temperature and the pH (Emerson et al., 1975). Specifically, FAN, which was suggested to be the most toxic form of ammonia (Massé et al., 2014), increases along with temperature and pH. Methanogenesis, the last step of AD process, is more sensitive to ammonia than hydrolysis, acidogenesis and acetogenesis steps (Yenigün and Demirel, 2013). Furthermore, in most of the studies, hydrogenotrophic methanogens were reported to be more robust to ammonia toxicity than aceticlastic methanogens (Schnürer et al., 1999; Werner et al., 2014; Dai et al., 2017). However, controversial results can also be found (Calli et al., 2005; Karakashev et al., 2005).

Considering ammonia inhibition is such a serious and highly debated topic, a great number of studies focusing on the impact of ammonia levels and on inhibition mechanism have been conducted in different reactor types (Angelidaki and Ahring, 1993; Sung and Liu, 2003; Cuetos et al., 2008; Wang et al., 2015; Chen et al., 2016). As a result, it is reviewed that

50% inhibition was caused by TAN concentrations ranging from 1700 to 14000 mg NH_4^+-N L^{-1} depending on different experimental conditions (Chen et al., 2008). However, in most of the aforementioned studies, ammonium chloride (NH_4Cl) was used as the inhibitor (ammonia source), and only few experiments can be found using other ammonia sources (Sterling et al., 2001; Westerholm et al., 2012; Dai et al., 2017). As a salt, NH_4Cl can dissociate immediately after addition into aqueous solutions and release chloride anions and ammonium cations, as shown in Eq. (2). However, since chloride anions could also be a potential inhibitor to AD process (Riffat and Krongthamchat, 2006; Viana et al., 2012), it is difficult to differentiate if the inhibitory effect only comes from ammonia. Moreover, in the real AD applications, when nitrogen-rich substrates are used as feedstocks, ammonia is usually formed by the degradation of proteins, urea and nucleic acids (Rajagopal et al., 2013). Furthermore, urea is the main part of animal urine besides water; thus abounds in animal slurry (e.g. poultry, mink pig, cattle) and slaughterhouse wastewater (Møller et al., 2004). Without urease, which is the enzyme that catalyses urea hydrolysis, urea in aqueous solutions has a negligible reaction rate constant of $6.3*10^{-9}$ s^{-1} and a half-life of 3.5 years (Krajewska, 2009). However, urease can be synthesized by different microorganisms, including some bacteria involved in AD process, which can accelerate the hydrolysis of urea by nearly 10^{14} times faster than the uncatalysed decomposition (Ciurli et al., 1999). As shown in Eq. (3), the direct hydrolysed product of urea is the most toxic ammonia form (i.e. FAN) (Zimmer, 2000). In addition, hydrolysis of urea causes sudden pH increase, which could negatively affect the AD process (Mobley et al., 1995; Ciurli et al., 1999).

$$NH_3\ (aq.) + H_2O\ (l.) \leftrightarrow NH_4^+\ (aq.) + OH^-\ (aq.) \quad \text{Eq. (1)}$$

$$NH_4Cl\ (s.) + H_2O\ (l.) \rightarrow NH_4^+\ (aq.) + Cl^-\ (aq.) \quad \text{Eq. (2)}$$

$$CO(NH_2)_2\ (s.) + 2H_2O\ (l.) \xrightarrow{urease} 2NH_3\ (aq.) + H_2CO_3\ (aq.) \quad \text{Eq. (3)}$$

Thus, in order to separate the inhibition only caused by ammonia and simulate this process closer to realistic conditions, urea was used as ammonia source in reactors fed with cattle manure (Sterling et al., 2001). However, among the limited studies using urea as ammonia source, nothing can be found about its effect on methanogens. Considering methanogenesis is the most sensitive step of AD process (Chen et al., 2008), it is important to understand the urea effect on different methanogens. In addition, to date, there are no studies assessing simultaneously the effect of NH_4Cl and urea on methanogenic archaea.

Therefore, the main aim of the present study was to investigate the effect of two different ammonia sources on four pure methanogenic strains (i.e. two aceticlastic and two hydrogenotrophic), under mesophilic (37°C) and thermophilic (55°C) conditions. To fulfil this aim, firstly, the effect on pH caused by the NH_4Cl dissociation and urea hydrolysis in AD batch reactors was investigated. Secondly, under controlled pH conditions (i.e. 7 and 8), five different TAN levels (i.e. ten different FAN levels) were applied on each pure methanogenic strain to evaluate the effect of the two ammonia sources on the cultures, independently of the pH.

2 Materials and methods

2.1 Pure strains, ammonia sources and enzyme

Four pure methanogenic strains (aceticlastic: *Methanosarcina thermophila* TM-1 DSM No.1825 and *Methanosarcina barkeri* MS DSM No. 800; hydrogenotrophic: *Methanoculleus thermophilus* CR-1 DSM No. 2373 and *Methanoculleus bourgensis* $MS2^T$ DSM No. 3045) were purchased from DSMZ GmbH Company and used throughout the study. *M. thermophila* and *M. thermophilus* are thermophilic, while *M. barkeri* and *M. bourgensis* are mesophilic methanogens. All the pure strains were cultivated in the specific growth media suggested by

DSMZ GmbH Company. Specifically, the growth media used were medium 120 (DSMZ, 2014a) for *M. thermophila*, medium 120a (DSMZ, 2014b) for *M. barkeri*, medium 141 (DSMZ, 2017) for *M. thermophilus*, and medium 332 (DSMZ, 2014c) for *M. bourgensis*. The carbon sources that were used for each strain were: acetate and methanol for *M. thermophila*; CO_2 for *M. thermophilus*; methanol for *M. barkeri*; and formate and CO_2 for *M. bourgensis*.

Ammonium chloride (Sigma-Aldrich, CAS no. 12125-02-9) and urea (Sigma-Aldrich, CAS no. 57-13-6) were used as ammonia sources for the main experiment. Urease (Type IX, Sigma-Aldrich, CAS no. 9002-13-5) from *Canavalia ensiformis* (jack bean) seeds was used as enzyme to hydrolyse urea. A buffer solution consisted of 0.2 M sodium phosphate with pH 7.3 was prepared for the dissolution of the enzyme before use.

2.2 Experimental setup

Two batch experimental assays were performed in this study to investigate the effect of different ammonia sources on pH fluctuation of the reactors (Assay I) and on the methanogenic process efficiency (Assay II). Before the experiments started, the pure strains, bought from DSMZ (DSMZ GmbH Company, Germany), were cultivated according to its corresponding cultivation protocols (DSMZ, 2014c; DSMZ, 2014b; DSMZ, 2014a; DSMZ, 2017). After several (4-6) generations, the cultures were used as inocula in the two experimental assays of the current study with a 20/80 (v/v) inoculum to medium ratio throughout the experiment. Meanwhile, urease was added to all batch reactors regardless of the ammonia source. Furthermore, all the experiments were conducted in triplicates.

2.2.1 Assay I: Effect on pH

All the pure strains were tested under different ammonia levels as depicted in Table 1. Serum vials were used with 40 and 118 mL working and total volume, respectively. After adding the corresponding medium, each vial was closed with butyl rubber stopper and sealed with aluminium caps, then flushed with a mixture gas of N_2/CO_2 (80/20, v/v) to create anoxic

conditions and autoclaved to provide sterile conditions. Other solutions that could not be autoclaved according to the instructions ($NaHCO_3$, Na_2CO_3, Vitamin, Methanol, L-cysteine-$HCl \cdot H_2O$ and $Na_2S \cdot 9H_2O$) were introduced by using sterilized, 0.2 μm pore size, Minisart® NML Syringe Filters (Sartorius Stedim Biotech GmbH, Germany) to avoid any contamination. $Na_2S \cdot 9H_2O$ solution was added as a reducing agent after inoculation. In addition, pure H_2 (62.4 mL) and CO_2 (15.6 mL) were added in the headspace of the batch reactors of the hydrogenotrophic strains. Afterwards, all the batch reactors were incubated at their corresponding temperatures (37±1°C for mesophilic and 55±1°C for thermophilic). The pH was measured after the urea hydrolysis finished (approximately 20 hours after the incubation stated based on preliminary hydrolysis test, and the details were provided in the E-supplement file).

2.2.2 Assay II: Effect on methanogenesis

In this assay, two different ammonia sources with five different TAN and ten different FAN levels (as shown in Table 2) were tested on all the methanogens. For all the strains, serum vials with 40 mL working volume was used, while total volume of 245 mL was used for *M. thermophila* and *M. thermophilus* cultivation, and total volume of 118 mL was used for *M. barkeri* and *M. bourgensis*. The reactors were closed with rubber stoppers, sealed with aluminium caps, and flushed with a mixture N_2/CO_2 gas (80/20, v/v) after the addition of medium. All the reactors containing medium were autoclaved before inoculation. Chemical solutions, which could not be autoclaved, were added through sterilized filters afterwards. In addition, for hydrogenotrophic *M. thermophilus* and *M. barkeri*, H_2/CO_2 (80/20, v/v) mixture gas was injected into the headspace of the reactor to form 1 bar overpressure. Furthermore, a pH adjustment strategy (the details were provided in the E-supplement file) was performed to ensure the same pH levels (7 and 8) for each individual experiment using 4 M HCl and/ or NaOH solutions. Specifically, for reactors with NH_4Cl, where the dissociation happened

immediately, pH adjustment was performed before the incubation started. However, for reactors containing urea and the hydrolysis happened slowly, the pH was adjusted several times until the hydrolysis finished (the details were provided in the E-supplement file). Finally, all the batch reactors were incubated in their corresponding temperatures (37±1^{o}C for mesophilic and 55±1^{o}C for thermophilic).

2.3 Analytical methods

Methane accumulation in the headspace of the batch reactors was determined by a gas chromatographer (Trace 1310 GC-TCD, Thermo Fisher, Denmark) equipped with a TracePLOT TG-BOND Q 26004–6030 column (30 m x 0.32 mm I.D., film thickness 10 μm) (Thermo Fisher), and helium was used as carrier gas (Tian et al., 2017). The pH of each reactor was measured with PHM99 LAB pH meter (Radiometer TM).

2.4 Calculations and statistics

2.4.1 Free ammonia

The free ammonia concentration was calculated based on the following equation (Siles et al., 2010):

$$FAN = \frac{TAN}{1+\frac{10^{-pH}}{K_a}} \quad \text{Eq. (1)}$$

where K_a is the dissociation constant affected by temperature, which equals to 1.29×10^{-9} and 3.91×10^{-9} in this study for mesophilic and thermophilic condition, respectively.

2.4.2 Methane production inhibition

The methane production inhibition was defined as the ratio of the difference between theoretical and practical methane production divided by the maximum theoretical methane production. Maximum theoretical production, for the different carbon sources in the medium, was calculated according to Angelidaki et al. (2011) and it was 122, 373 and 525 mL $CH_4 \cdot g^{-1}$

VS for formate, acetate and methanol. Meanwhile, for the H_2/CO_2 mixture gas, it was calculated based on that 1 mL CH_4 forms from 4 mL H_2 and 1 mL CO_2.

2.4.3 Maximum specific growth rate

Maximum specific growth rate (μ_{max}) was calculated through the OriginLab program (OriginLab Corporation, Northampton, Massachusetts) by calculating the slope of the linear part of the semi-logarithmic graph of the methane production of the reactors versus time (Gray et al., 2009).

2.4.4 Statistical analysis

The OriginLab program was used for statistical analyses and data plotting. One-way and two-way ANOVA were used to evaluate the statistically differences ($p<0.05$) of ammonia inhibition under different parameters (e.g. different ammonia sources, ammonia levels and pH levels). Single outliers test was applied to the triplicate measurements if needed.

3 Results and discussion

3.1 Impact on pH from two different ammonia sources

The impact of urea hydrolysis and NH_4Cl dissociation on pH was significantly different ($p<0.05$, Fig. 1). Specifically, after urea hydrolysis completed, except for the basic TAN levels, the pH increased to around 9 for *M. thermophila*, *M. barkeri*, and *M. bourgensis*, which was outside of the pH limits (6.5-8.5) for AD process (Lay et al., 1998). This increase in pH after urea hydrolysis, was in agreement with a previous study (Udert et al., 2003) where elevated pH was observed alongside the extent of urea hydrolysis. The pH of *M. thermophilus* increased alongside the urea concentration, and it was about 8.5 at the highest TAN level (5000 mg NH_4^+-N·L^{-1}). This different performance of *M. thermophilus* from the other strains could be explained by the stronger buffer capacity in *M. thermophilus* medium compared to the other media due to the higher $NaHCO_3$ concentration. In contrast, NH_4Cl dissociation did

not have any significant effect on the pH of batch reactors, with a maximum pH drop of approximately 0.3 units at the highest TAN levels (10000 mg NH_4^+-N·L^{-1}). Therefore, it seems that NH_4Cl is not a representative ammonia source to simulate ammonia inhibition in AD reactors because, contrary to urea, it does not have an analogous pH effect.

Meanwhile, it also can be seen that a medium with strong buffer capacity could mitigate the effect of urea hydrolysis on pH (e.g. *M. thermophilus* case); thus, it is reasonable to suspect that the pH of manure-based AD reactors (high buffer capacity) would not increase in such a great extent. At the same time, without pH adjustment, the pure strains are not expected to grow with urea (except in the basic TAN concentrations), due to the unfavourable pH levels (> 8.5). Therefore, all the following methanogenesis batch experiments in assay II, were designed with a pH adjustment strategy (adjust the initial pH level to 7 and 8, respectively) to compare the effect of the two different ammonia sources on the pure methanogenic strains, independently of the pH.

3.2 Methanogenesis performance of different methanogens

3.2.1 Aceticlastic *M. thermophile* and *M. barkeri*

Urea had similar or significantly higher (p<0.05) inhibitory effect on both aceticlastic strains compared to NH_4Cl in the majority of the tested TAN levels. For example, NH_4Cl inhibited the methane production of *M. thermophila* by 58% at 5000 mg NH_4^+-N·L^{-1} (pH=8); at the same time, urea inhibited the same strain more than 90% at 5000 mg NH_4^+-N·L^{-1} for pH=7 and at all TAN levels above 3000 mg NH_4^+-N·L^{-1} for pH=8 (Fig. 2a). The different inhibition effects were also reflected on the longer lag phases at the same ammonia levels for urea compared to NH_4Cl. To be specific, up to threefold longer lag phase periods were in urea reactors compared to NH_4Cl reactors (Table 3). Furthermore, at lower FAN levels (< 151 mg NH_3-N·L^{-1}), μ_{max} of *M. thermophila* was between 0.04-0.06 h^{-1} for both urea and NH_4Cl reactors coinciding with μ_{max} values reported before (Sowers et al., 1984; Mladenovska and

Ahring, 2000). However, NH_4Cl reactors had significantly higher μ_{max} compared to urea reactor for FAN levels above 151 mg NH_3-N·L^{-1}, which indicates a stronger inhibitory effect of urea (Fig. 2c).

M. barkeri was the most sensitive methanogenic strain to ammonia compared to all the other tested strains. Almost 100% inhibition was observed at 64 (5000 mg NH_4^+-N·L^{-1}, pH=7) and 89 mg NH_3-N·L^{-1} (7000 mg NH_4^+-N·L^{-1}, pH=7) for reactors with urea and with NH_4Cl, respectively (Fig.2b). These results were in accordance to previous studies reporting 50% inhibition of *M. barkeri* growth at 42 mg NH_3-N·L^{-1} and more than 95% inhibition at 88 mg NH_3-N·L^{-1} (Sprott and Patel, 1986; Hajarnis and Ranade, 1993). However, although complete inhibition occurred in most ammonia levels, for FAN levels lower than 64 mg NH_3-N·L^{-1}, where methanogenesis was observed, urea was clearly stronger inhibitor than NH_4Cl. Furthermore, urea prolonged the lag phase up to fourfold compared to NH_4Cl (Table 3). Even though *M. barkeri* was the most sensitive methanogenic strain tested in the present study, it had the highest μ_{max} of 0.11-0.12 h^{-1} (optimal conditions), which decreased alongside with the increase of ammonia levels (Fig. 2d). Similar specific growth rates (0.10-0.14 h^{-1}) of *M. barkeri* were reported by Jarrell et al. (1987) when TAN was below 1.4 NH_4^+-N·L^{-1}, and more than 50% reduction was detected around 4 NH_4^+-N·L^{-1}. However, no significant difference (p>0.05) of the μ_{max} can be found between urea and NH_4Cl reactors.

3.2.2 Hydrogenotrophic *M. thermophilus* and *M. bourgensis*

Overall, hydrogenotrophic methanogens were, as expected (Werner et al., 2014), more tolerant to NH_4Cl than the aceticlastic methanogens tested in the current study. Interestingly, it was also found that hydrogenotrophic methanogens were more tolerant to urea than aceticlastic methanogens. Nevertheless, similar to aceticlastic strains, urea also had a higher inhibitory effect on the hydrogenotrophic methanogens compared to NH_4Cl. However, there was an exception for *M. thermophilus* at low TAN levels (< 3000 mg NH_4^+-N·L^{-1}), where

NH_4Cl seemed to be more toxic than urea (Fig. 3a). The reasons might be firstly, the pH of the urea reactors did not increase due to the strong buffer capacity of *M. thermophilus* medium as discussed previously; Secondly, NH_4Cl reactors suffered higher toxicity than urea reactors at the beginning because of the higher ammonia concentration from instant NH_4Cl dissociation compared to from the gradual urea hydrolysis process. However, at higher TAN levels (> 3000 mg NH_4^+-N·L^{-1}), urea inhibited *M. thermophilus* significantly stronger ($p<0.05$) than NH_4Cl. All the *M. thermophilus* reactors had a lag phase smaller than 1.2 days (Table 4) maintaining a μ_{max} between 0.03-0.04 h^{-1} indicating that *M. thermophilus* was able to cope with the strong ammonia toxicity. This was in agreement with Wang et al. (2015) reporting no significant drop ($p>0.05$) on methane production at ammonia levels up to 7000 mg NH_4^+-N·L^{-1} for *M. thermophilus* with a μ_{max} around 0.03 h^{-1}.

M. bourgensis was the most ammonia tolerant methanogenic strain tested in the current study, in which no more than 15% inhibition was observed, and independently of the ammonia sources, ammonia levels and pH levels (Fig.3b). This high tolerance was expected because *M. bourgensis* was reported (Fotidis et al., 2014) to thrive under high ammonia levels (5000 mg NH_4^+-N L^{-1}). Moreover, Westerholm et al. (2015) observed that *M. bourgensis* was the dominant archaeon in AD reactors operated under high FAN levels (900 mg NH_3-N·L^{-1}), and Wang et al. (2015) also demonstrated that *M. bourgensis* can work properly at TAN levels up to 7000 mg NH_4^+-N·L^{-1}. However, even with this tolerant methanogen, urea was proven more toxic than NH_4Cl, resulting in lag phases up to ten days for TAN levels above 5000 mg NH_4^+-N·L^{-1} (pH 8), compared to only two days lag phase for the NH_4Cl at the highest TAN levels. The same trend was observed among the specific growth rates, with significantly lower μ_{max} for the urea reactors compared to NH_4Cl reactors in majority of the tested ammonia levels.

3.3 The ammonia sources and the inhibition mechanism

In general, urea was a significantly stronger inhibitor than NH_4Cl (Table 5). This could be explained by the different manners that urea and NH_4Cl introduce TAN and FAN into the reactors. Specifically, NH_4Cl, as an easily soluble salt, can fully dissociate in aqueous phase immediately after its addition and the direct dissociative products are ammonium ions (Eq. 2), instead of the more toxic FAN form (Massé et al., 2014). On the contrary, urea, which is an organic compound, can only be hydrolysed slowly with the presence of urease, and produce directly FAN (Eq. (3)), which is the most toxic ammonia form (Zimmer, 2000). Therefore, relatively high FAN levels develop instantly after urea hydrolysis, before the final $NH_4^+ \Leftrightarrow NH_3$ equilibrium (Eq. 1) is established, driven by the pH and the temperature (Emerson et al., 1975). Compared to low FAN levels after NH_4Cl dissociation, this momentary exposure of the methanogenic cells to such high FAN concentrations after urea hydrolysis, could have a greater impact in their metabolic activity. Furthermore, NH_4Cl dissociation does not have a significant effect on the pH of the reactor and thus does not create unfavourable pH conditions for the methanogens. On contrary, urea hydrolysis without pH control could increase the pH of the reactor into unfavourable levels. Even though pH was adjusted constantly in the current experiment, until the hydrolysis of urea was completed, it was impossible to avoid a temporal pH increase during the urea hydrolysis period (details are provided in the E-supplement file). Thus the combined effect of momentary high FAN concentrations and pH increase, even for short time periods during the hydrolysis phase, is proposed as the main mechanism for the stronger inhibitory effect of urea compared to NH_4Cl on the pure methanogenic strains tested in this study.

4 Conclusions

The current study demonstrated that urea was significantly more toxic compared to NH_4Cl during AD process. Furthermore, urea hydrolysis resulted in a great pH increase to

unfavourable levels for methanogenic growth. However, a high buffer capacity can mitigate the pH increase and lower the ammonia toxicity from urea. Additionally, hydrogenotrophic methanogens were more tolerant, not only to NH_4Cl but also to urea, compared to aceticlastic methanogens. Finally, considering only pure strains were tested in this study, further studies in a more complex environment of real AD digesters are still needed to analyse the inhibition effect of urea.

Appendix A. Supplementary material

E-supplementary data for this work can be found in e-version of this paper online: Fig. S1. Preliminary urea hydrolysis test at different ammonia and pH levels with/ without urease under two different incubation temperatures, a) for thermophilic *M. thermophila* and b) for mesophilic *M. bourgensis*. Fig. S2. pH adjustment strategies to 7 and 8 at different urea concentrations for a) *M. thermophila*, b) *M. barkeri*, c) *M. thermophilus*, d) *M. bourgensis*

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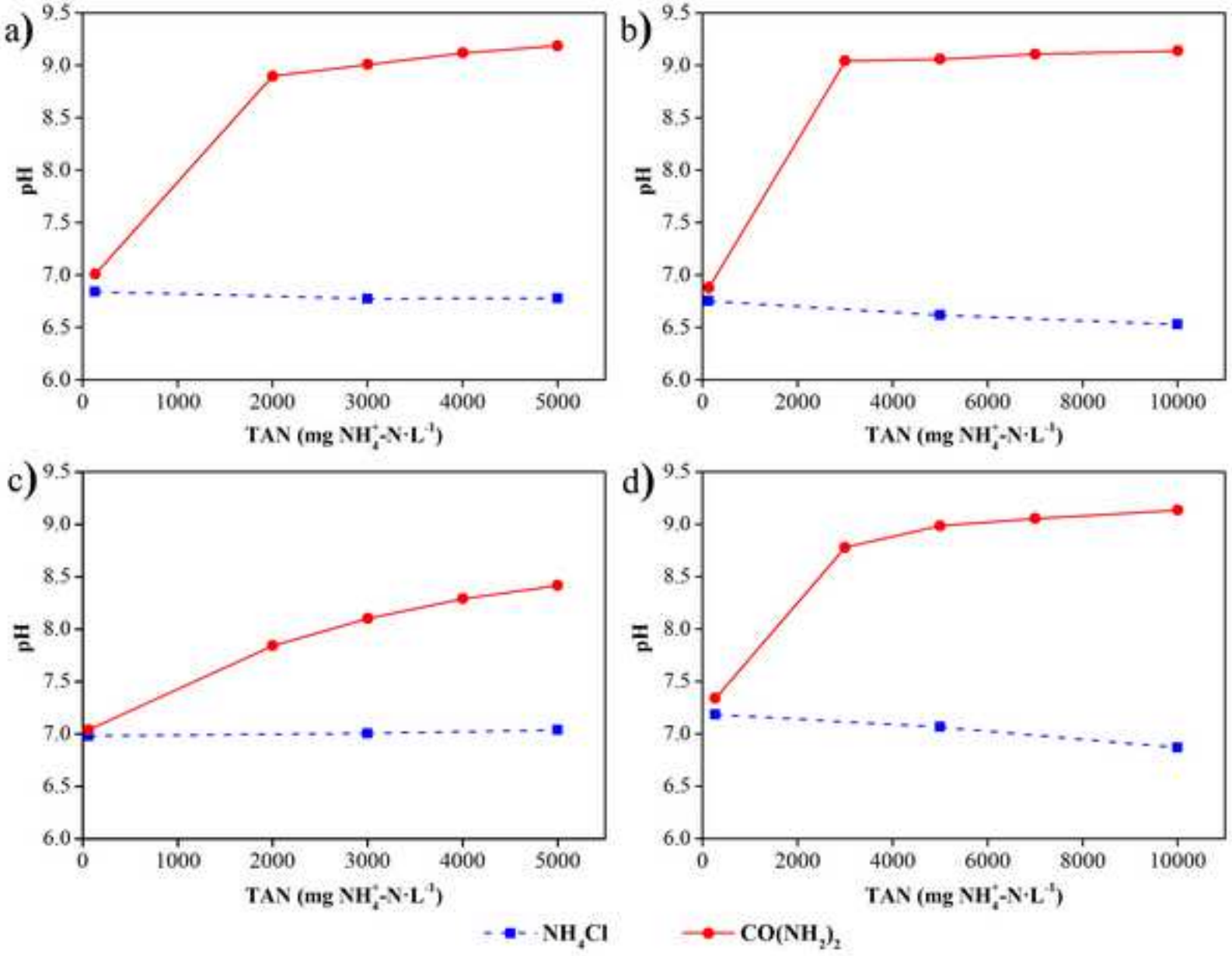
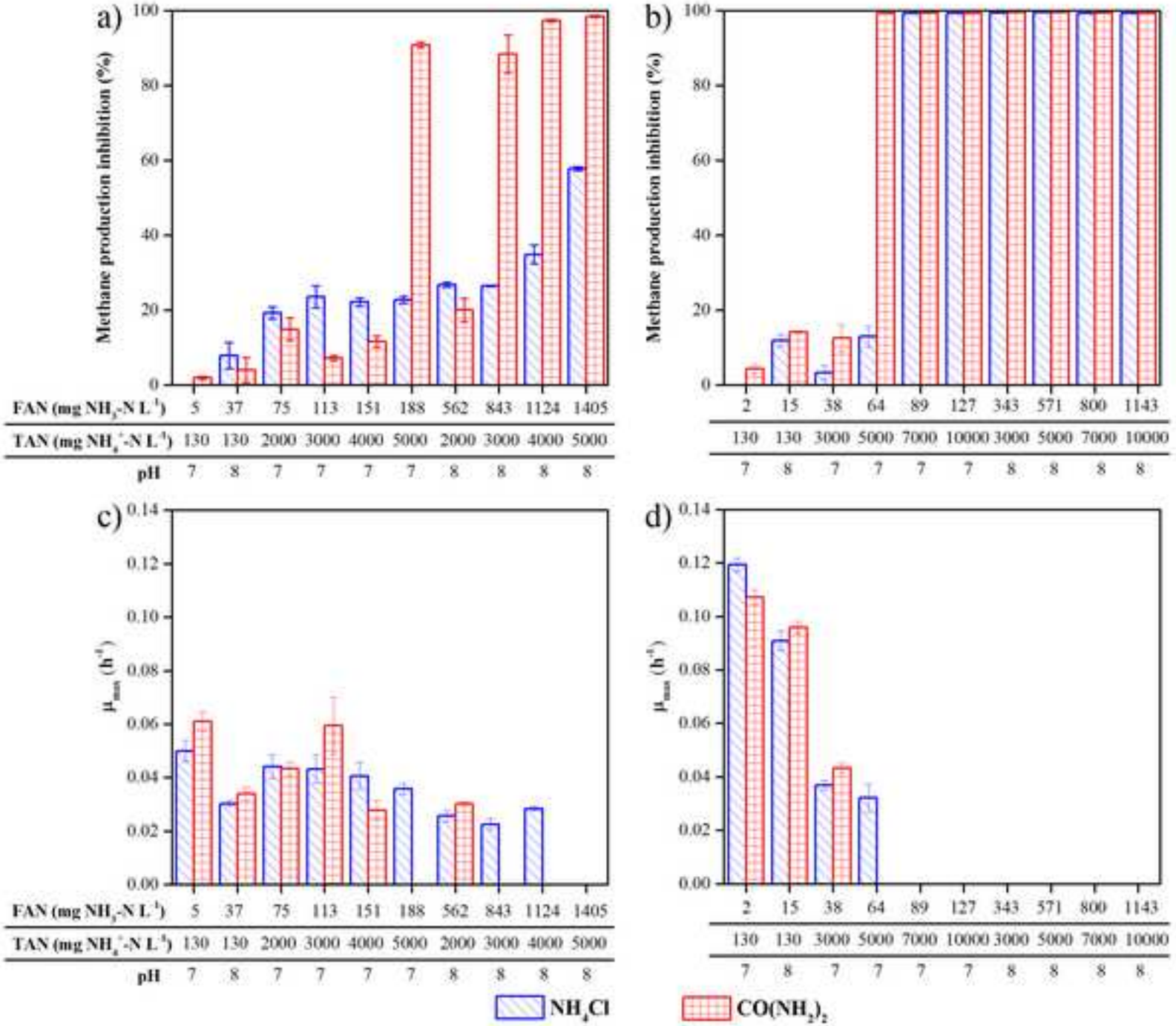
Figure legends

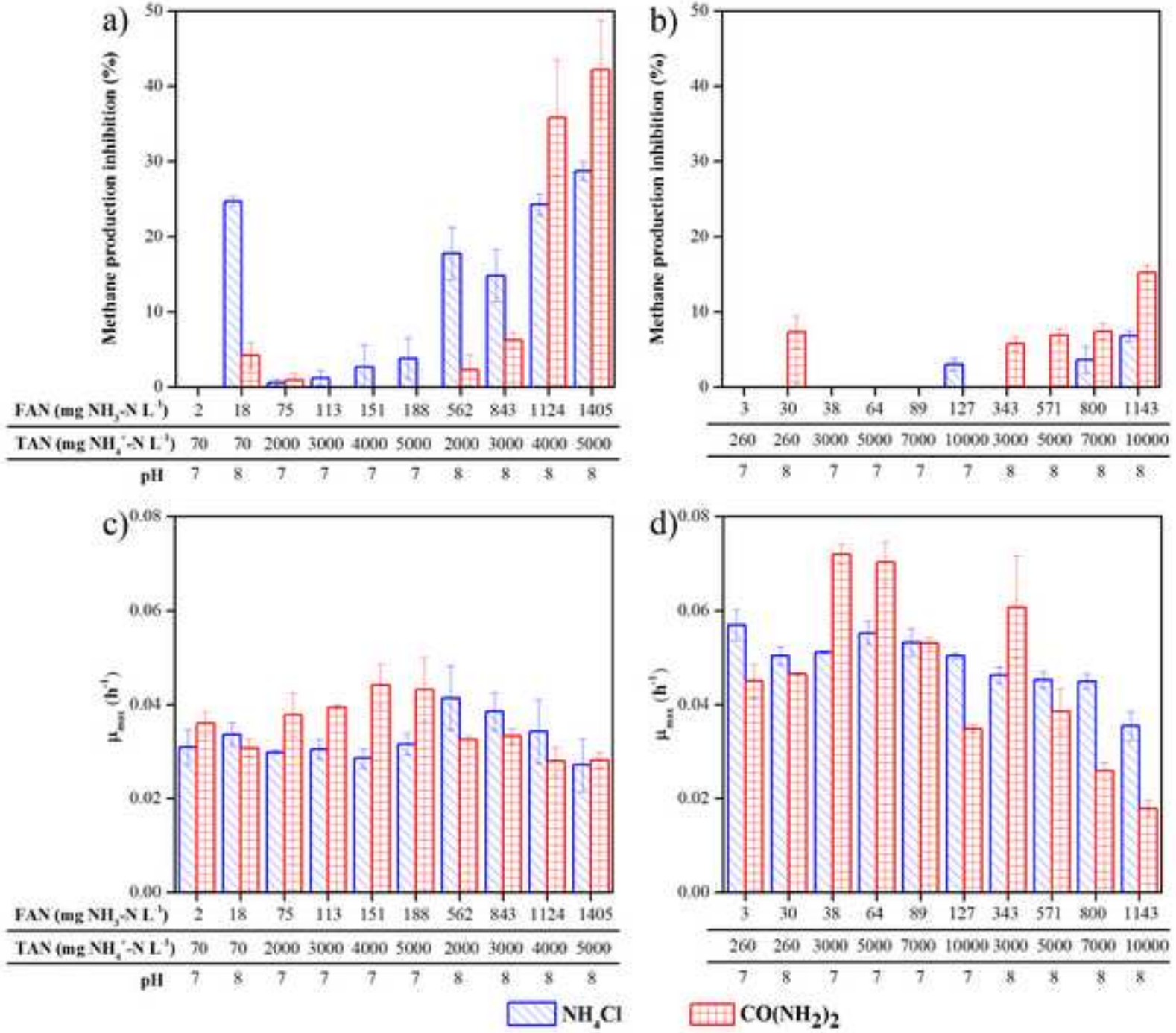
Fig. 1. pH value after the hydrolysis of the urea and the dissolution of the NH_4Cl at different ammonia levels, a) *M. thermophila*, b) *M. barkeri*, c) *M. thermophilus*, d) *M. bourgensis*

Fig. 2. Final methane production inhibition and μ_{max} of *M. thermophila* and *M. barkeri* under different ammonia sources, ammonia levels and pH levels, a) inhibition of *M. thermophila*, b) inhibition of *M. barkeri*, c) μ_{max} of *M. thermophila*, d) μ_{max} of *M. barkeri*.

Fig. 3. Final methane production inhibition and μ_{max} of *M. thermophilus* and *M. bourgensis* under different ammonia sources, ammonia levels and pH levels, a) inhibition of *M. thermophilus*, b) inhibition of *M. bourgensis*, c) μ_{max} of *M. thermophilus*, d) μ_{max} of *M. bourgensis*.







445 **Table 1.** Different ammonia levels for the two ammonia sources in Assay I.

Strains	**Ammonia sources**	**TAN (mg NH_4^+-N·L^{-1}) ***
M. thermophila	$CO(NH_2)_2$	130, 2000, 3000, 4000 and 5000
	NH_4Cl	130, 3000 and 5000
M. barkeri	$CO(NH_2)_2$	130, 3000, 5000, 7000 and 10000
	NH_4Cl	130, 5000 and 10000
M. thermophilus	$CO(NH_2)_2$	70, 2000, 3000, 4000 and 5000
	NH_4Cl	70, 3000 and 5000
M. bourgensis	$CO(NH_2)_2$	260, 3000, 5000, 7000 and 10000
	NH_4Cl	260, 5000 and 10000

446 * The lowest TAN level is the basic ammonia levels of the medium.

447

448 **Table 2**. Different ammonia and pH levels under the two different ammonia sources of Assay

449 II.

Strains	TAN (mg NH_4^+-N·L^{-1}) *	Ammonia sources	pH levels
M. thermophila	130, 2000, 3000, 4000 and 5000	NH_4Cl, $CO(NH_2)_2$	7, 8
M. barkeri	130, 3000, 5000, 7000 and 10000	NH_4Cl, $CO(NH_2)_2$	7, 8
M. thermophilus	70, 2000, 3000, 4000 and 5000	NH_4Cl, $CO(NH_2)_2$	7, 8
M. bourgensis	260, 3000, 5000, 7000 and 10000	NH_4Cl, $CO(NH_2)_2$	7, 8

450 * The lowest TAN level is the basic ammonia levels of the medium.

451

452 **Table 3**. Lag phase (days) of *M. thermophila* and *M. barkeri* under different experimental

453 conditions.

Strains	**Ammonia sources**	**pH**	**TAN levels (mg NH_4^+-N·L^{-1})**				
			130 (130) *	2000 (3000)	3000 (5000)	4000 (7000)	5000 (10000)
M. thermophila	**NH_4Cl**	7	0	0	0	0	0
		8	7.0 ± 3.0	11.0 ± 6.2	17.5 ± 7.5	32.6 ± 7.6	ND **
	$CO(NH_2)_2$	7	0	0	3.6 ± 0.5	4.4 ± 0.5	ND
		8	3.6 ± 1.9	33.0 ± 6.2	ND	ND	ND
M. barkeri	**NH_4Cl**	7	1.0	6.9	32.8 ± 5.9	ND	ND
		8	0.9	ND	ND	ND	ND
	$CO(NH_2)_2$	7	1.1	24.8 ± 8.0	ND	ND	ND
		8	1.2	ND	ND	ND	ND

454 *Numbers outside parentheses were ammonia concentrations for *M. thermophila*, and the ones inside for *M.*

455 *barkeri*.

456 ** ND: Not defined.

457

458

459 **Table 4**. Lag phase (days) of *M. thermophilus* and *M. bourgensis* under different experimental
460 situation.

Strains	Ammonia sources	pH	TAN levels (mg NH_4^+-N·L^{-1})				
			70 (260)*	2000 (3000)	3000 (5000)	4000 (7000)	5000 (10000)
M. thermophilus	NH_4Cl	7	0	0	0	0	0
		8	0	1.2 ± 0.5	1.2 ± 0.5	1.2 ± 0.8	0.9 ± 0.7
	$CO(NH_2)_2$	7	0	0	0	0	0
		8	0	0	0	0	0
M. bourgensis	NH_4Cl	7	0	0	0	0	0
		8	0	0	0	0	2.0
	$CO(NH_2)_2$	7	0	0	0	0	0
		8	0	1.0	2.7 ± 0.5	4.3	10.1

461 *Numbers outside parentheses were the ammonia concentrations for *M. thermophilus*, and the ones inside for *M.*
462 *bourgensis*.
463

464 **Table 5.** Overall comparison of highest methane production inhibition of all strains.

Strains	pH	NH_4Cl	$CO(NH_2)_2$
M. thermophila *	7	22.9 ± 0.9 %	91.0 ± 0.8 %
	8	57.9 ± 0.5%	98.5 ± 0.2 %
M. barkeri **	7	99.4 ± 0 %	99.4 ± 0.1 %
	8	99.5 ± 0 %	99.6 ± 0.1 %
M. thermophilus *	7	3.8 ± 2.7 %	0%
	8	28.7 ± 1.2 %	42.2 ± 6.6 %
M. bourgensis *	7	3.1 ± 0.8 %	28.7 ± 1.2 %
	8	6.8 ± 0.7 %	15.2 ± 1.0 %

465 * Detected under the highest ammonia levels, specifically, for both pH levels, 5000 mg NH_4^+-N·L^{-1} for *M.*
466 *thermophila* and *M. thermophilus*, and 10000 mg NH_4^+-N·L^{-1} for *M. bourgensis*.
467 ** Detected under a relatively low ammonia levels, specifically, 7000 and 5000 mg NH_4^+-N·L^{-1} at pH 7 for
468 NH_4Cl and urea, respectively, and 3000 mg NH_4^+-N·L^{-1} at pH 8 for both.
469

Highlights

- Urea hydrolysis increases reactor pH significantly more than ammonium chloride
- Urea is more toxic to methanogenic archaea than ammonium chloride
- Combined high free ammonia and pH levels is the toxicity mechanism of urea
- Hydrogenotrophic methanogens are more robust than aceticlastic methanogens to urea